HUMAN LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN-1 (LFA-1): IDENTIFICATION OF MULTIPLE ANTIGENIC EPITOPES AND THEIR RELATIONSHIP TO CTL-MEDIATED CYTOTOXICITY

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Abstract

Cytotoxic T lymphocytes (CTL) play an important role in the immune response to cell surface displayed antigens such as those found on virus-infected cells, tumor cells or histoincompatible tissue grafts. Extensive studies on the cellular mechanism involved in the killing reaction have indicated that lysis is a complex multi-step process resolvable into two discrete cation-requiring phases (1-4). A new approach to identifying the molecular components of the cytolytic attack mechanism has been to employ anti-CTL monoclonal antibodies (MAb) capable of inhibiting cytosis in the absence of complement as specific probes for the identification and characterization of function-associated molecules (5-8). The validity of this approach is based largely on the fact that MAb to a variety of other CTL surface structures do not block cytosis, thus, inferring that inhibitory MAb recognize specific function-associated antigens (9).

Several monoclonal antibodies that recognize molecules on cells or T cell subpopulations have been shown to block cytolytic activity in the absence of complement. In man, OKT3 or Leu 4 (T3) MAb recognizes a complex glycoprotein(s) of 20,000 (10-13). The antigen defined by the MAb OKT8, Leu2a, or E series (T8) is a 30-32,000 and 43,000 Mr disulfide linked glycoprotein complex(s) (11, 14-16). The OKT4 or Leu 3 antigen (T4) is 55,000 Mr (17, 18). Murine CTL are inhibited by MAb against the Lyt-2,3 complex (19-23) (the structural homologue of antigen (24)) and to the lymphocyte function-associated antigen 1 (LFA-1), a 180,000-95,000 Mr heterodimer (9, 25, 26).

In a recent report, several mouse hybridoma cell lines secreting MAb that blocked the cytolytic activity of HLADR specific human CTL were isolated (8). These MAb identified the novel human CTL surface structures and were designated lymphocyte function-associated antigens (LFA-1), 2 and 3. Human LFA-1, a non-disulfide linked heterodimeric lymphocyte surface structure, appears to be the structural homologue of the previous described mouse LFA-1 molecule. Functional studies on mouse LFA-1 have implicated its role in the initial recognition-attachment phase of the CTL reaction but it is probably distinct from the CTL antigen-specific receptor (5). Anti-LFA-2 MAb precipitated a 49,000 Mr component. Anti-LFA-3 MAb recognized a 60,000 Mr protein on a human alloantigen specific CTL line.

In the present report the topographic distribution of antigen epitopes on the human and mouse LFA-1 molecules, the qualitative expression of cell surface human LFA-1 relative to other CTL membrane antigens and the quantitative effects of the anti-LFA-1 MAb on a Class I allospecific human CTL line have been investigated. The results suggest the presence of several unique antigenic epitopes on the human LFA-1 molecule that reside with varying degrees of spatial proximity to the functional region(s) of the LFA-1 molecule.

MATERIALS AND METHODS

Monoclonal antibodies. Two series of mouse anti-human LFA-1 monoclonal antibodies were obtained from hybrid cell lines produced by fusion of human CTL-immune Balb/c spleen cells with the P3X63 Ag8.653 (TS1) series, NS1 (TS2 series) myeloma cell lines as previously described (6). Both sets of hybridoma cell lines have been subcloned twice and are stable. T antibodies secreted by the TS1 and TS2 series are of the IgG1, K ch. subclass. Monoclonal antibody concentration in culture fluids was determined by single radial immunodiffusion (27) using a purified mouse IgG1, MAb (PA...
by Kolb (40) were used to directly immunoprecipitate LFA-1 and HLA. The immune complex adsorbed bacteria were washed three times in 10 mM TRIS with extraction buffer; 2) 10^9 bacteria cells were prewashed three times in warm PBS and were radiolabeled with homologous MAb. Nonspecific binding was subtracted before calculations. Incubation was continued for 45-60 min on ice. Controls for nonspecific inhibition of binding was performed with [3H]~-leucine-labeled or MAb as previously described (25, 27). The incubation was continued for 30 min with shaking. The washed immunoadsorbed antigens were eluted with SDS-PAGE sample buffer, and electrophoresed on 10% polyacrylamide slab gels (41). Autoradiography was performed on dried gels using intensifying screens (Kronex, DuPont, Wilmington, DE) for 48 hr at -85°C (42).

The TSl/18.1.1 anti-LFA-1 and the W6/32 and BBM.l MAb were purified by saturation and gel filtration chromatography (14). Analysis of IgG preparations by Na dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7% acrylamide) indicated that they were >98% pure IgG.

Purified monoclonal antibodies were radioiodinated with Na 125I (New England Nuclear, Boston, MA), specific activity 17 Ci/mg, using lodogen (33) to specific activities of 0.5 to 2 x 10^6 cpm/pg and were stored in 10% PBS-BSA-PBS at 4°C. A detergent extract was obtained by sonicating the radiolabeled cells in 50 mM TRIS with extraction buffer, 200 μg/ml BSA-PBS in microtiter plates for 60 min on ice. Cells were then washed four times with 200 μg/ml BSA-PBS by repeated centrifugation at 1000 x G for 2 min. Cells were transferred to vials and lyzed by the addition of 100 μl 0.1% NP-40 nonionic detergent solution and 3 ml liquid scintillation cocktail (Biofluor, New England Nuclear, Boston MA) was added for counting. The percentage of inhibition of binding was calculated as a ratio of the antibody bound in the presence of inhibitor to amount bound in PBSX33 culture supernatant. Generally, 1000-2000 cpm were bound for each labeled MAb in absence of any inhibitor. The nonspecific background binding was defined as counts per minute bound in the presence of unlabeled anti-LFA-1 MAb (50 μg/ml) and were subtracted from each sample to determine specific activity. Specific binding was subtracted before calculations.

**RESULTS**

**Human LFA-1 expresses multiple antigenic epitopes.** Isolation and characterization of seven stable mouse hydridoma cell lines secreting MAb (all of the γ1 subclass) that precipitated a noncovalently associated heterodimer of 177,000 and 95,000 Mr from human T and B lymphocytes have been reported (8). The topographical relationship of the antigenic epitope(s) recognized by this series of anti-human LFA-1 MAb was investigated with a competitive cross-inhibition binding assay using [3H]Igeucine bio-synthetically labeled MAb (Fig. 1). Two clearly distinct and unique epitopes were defined by MAb TS1/18 and TS2/14 since only the homologous antibody could act as a competitive inhibitor. Another distinct epitope was defined by the TS1/12 and TS1/11 MAb which acted as reciprocal competitive inhibitors. Monoclonal antibodies TS2/4, TS2/6, and TS1/22 defined complex partially overlapping antigenic epitopes on the human LFA-1 molecule. TS2/14 was a nonreciprocal inhibitor of the TS1/22 MAb. W6/32 anti-HLA did not inhibit the binding of any of the anti-LFA-1 MAb.

To confirm that the unique epitopes defined by these MAb

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was repeated to ensure complete removal of the antigens from the extracts. The other anti-LFA-1 and BBM.1 MAb were then used to immunoprecipitate their antigens from the precleared extracts before analysis of the immunoprecipitates by SDS-PAGE and autoradiography (Fig. 2). The TS1/18-4BCL quantitatively removed all of the LFA-1 from the extract (Fig. 2, lanes 1 and 2) as well as antigens that were reactive with all the other anti-LFA-1 MAb (lanes 3–8) without removing BBM.1 antigen (lane 9). Similarly the BBM.1-4BCL quantitatively removed all $\beta_2$-microglobulin (as HLA) without affecting the ability of anti-LFA-1 MAb to immunoprecipitate LFA-1. Thus, the unique epitopes defined by this collection of anti-LFA-1 MAb reside on the same molecule as that bound by the TS1/18 MAb.

Several rat MAb reactive with mouse LFA-1 (M17/4, M7/14, M17/7, and M17/5) were investigated for the topographic distribution of their epitopes on the mouse LFA-1 molecule (Fig. 3). The results indicated that M17/4, M17/5, M17/4 and M17/7 were efficient reciprocal competitive inhibitors of each other and thus define a common epitope. The M15/15 MAb was a partial competitive inhibitor of M17/4 and failed to compete with the others indicating M15/5 defined a partially shared but distinct epitope. Thus, of the rat MAb currently available, two partially related epitopes were recognized on the LFA-1 molecule.

Quantitation of cell surface LFA-1. The TS1/18 anti-human LFA-1 MAb was purified, iodinated, and used as a probe to determine the number of antigen binding sites per cell on a functional CTL line using a direct saturation binding assay. $^{125}$I- TS1/18 and $^{125}$I-W6/32 binding to CTL-11 was saturable and highly specific (Fig. 4). In six separate experiments human CTL-11 expressed 0.7 to 1.6 $\times 10^5$ LFA-1 sites/cell (mean 1.03 $\times 10^5$). Long term cultures (greater than 2 weeks) of CTL-11 expressed higher levels of LFA-1 than CTL-11 cultured for only 1–2 weeks. HLA-A, B, C antigenic sites were expressed at a 10-fold higher level than LFA-1 on the same cells ranging from 5.2 to 17 $\times 10^5$ sites/cell (mean 9.8 $\times 10^5$). Quantitative expression of LFA-1 and HLA-A, B, C on several
human cell lines and peripheral blood lymphocytes (PBL) (Table I) showed a relatively constant ratio of HLA to LFA-1 of almost 10, with the exception of the Epstein-Barr virus transformed JY B lymphoblastoid line which displayed 10-fold enhanced expression of HLA-A, B, C antigens (46). Analysis of CTL-11 by fluorescence activated flow cytometry (43, 44) (Materials and Methods) indicated LFA-1 was expressed on ≥98% of the cells. The T3, T8, and T4 antigens were expressed on 93%, 33%, and 70% of the cells, respectively.

Functional topography of the LFA-1 epitopes. The ability of an antibody to sterically inhibit the biological activity of a protein is thought to be related to the proximity or spatial organization of the antigenic epitope to the functional site(s) of the protein. This series of anti-LFA-1 MAb, although selected on the basis of their ability to inhibit human CTL activity, showed considerable variation in blocking cytolysis. Titration of each anti-LFA-1 MAb on the HLA-B7 specific CTL-11 line (Fig. 5) revealed significant and reproducible differences in the inhibitory activity of these anti-LFA-1 MAb. The TS1/18 MAb showed the greatest level of inhibition with 50% maximum inhibition of lysis occurring at 0.5–0.7 µg/ml; TS1/22 and TS2/6 were moderate inhibitors requiring 1–2 µg/ml to achieve 50% maximum inhibition; TS1/11 and TS2/14 inhibited lysis at high concentrations (>10 µg/ml) of MAb. In contrast, TS2/4 showed no inhibitory activity (less than 10%) in this particular assay. An identical pattern of reactivity with these same MAb has been observed with T4+ HLA-DR-specific human CTL (8). This indicated the variability in the degree of inhibition was not due to the antigen specificity of the CTL effectors. From the quantitative binding analyses presented earlier (Fig. 4) all antibodies were in considerable excess (10- to 50-fold) required for saturation of LFA-1 sites. This result suggested that the antigenic epitopes defined by these anti-LFA-1 MAb may be associated with unique functional regions on the LFA-1 molecule.

A comparison of the CTL-inhibiting activities of the anti-LFA-1 MAb with their epitope reactivities indicated that a complex relationship between the antigenic epitopes and the functional region(s) on the LFA-1 molecule (Table II). The TS1/18 MAb, which defined a unique epitope, showed the strongest inhibition of CTL activity. A moderate CTL-inhibiting MAb, TS2/6, defined an antigenic epitope partially related to a strongly (TS1/18) and a weakly (TS1/11) CTL-inhibiting MAb. In contrast, TS2/4, a non-CTL-inhibitory MAb, shares partial reactivity with antigenic epitopes exhibiting strong (TS1/18), moderate (TS1/22 and TS2/6), and weak (TS1/11) associations with CTL activity. The TS2/14 MAb, which defines a unique epitope, but also non-reciprocally cross inhibited binding of TS1/22, inhibited cytolysis to essentially the same degree as TS2/6 and TS1/22.

The rat anti-mouse LFA-1 MAb (M7 and M17 series) which recognize a common epitope, have previously been shown to inhibit mouse CTL function by 80–100%. In contrast, the anti-mouse LFA-1 MAb, M15/15, which recognized a partially related or different epitope, inhibited CTL killing by 50% (6, 31).

Anti-LFA-1 reversibly inhibits cytolysis. A moderate fluctuation in the inhibitory activity of TS1/18 was observed in several experiments. Since the variation may have been linked to the length of the CTL assay the effects of TS1/18 on the kinetics of CTL-11 mediated lysis of MST target cells was studied. CTL-11 was mixed in 100 µl medium containing 3 µg TS1/18.1.1 or 2A2 control MAb. 51Cr-labeled MST target cells were added to the mixture and the plates were centrifuged to start the reaction and were incubated at 37°C for various lengths of time. The results indicated TS1/18 MAb-treated cells showed a significant delay (1.5–2 hr) in the initial onset of target cytolysis with a subsequent increase in the rate of cytolysis to 10% lysis/hr (through the first 4 hr), approaching the rate observed in the untreated control.
The data represent the mean ± SD of triplicate wells.

**TABLE I**

Quantitation of LFA-1 and HLA-A,B,C sites on various cell types

<table>
<thead>
<tr>
<th>Cell</th>
<th>cpm Bound at saturation x 10^3</th>
<th>Sites/cell x 10^3</th>
<th>cpm Bound at saturation x 10^3</th>
<th>Sites/cell x 10^3</th>
<th>Ratio HLA/LFA-1</th>
</tr>
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<tbody>
<tr>
<td>CTL-11</td>
<td>9.35 ± 0.05</td>
<td>0.88</td>
<td>33.0 ± 0.19</td>
<td>9.5</td>
<td>10.6</td>
</tr>
<tr>
<td>PBL</td>
<td>3.64 ± 0.28</td>
<td>0.34</td>
<td>7.2 ± 0.02</td>
<td>2.1</td>
<td>6.2</td>
</tr>
<tr>
<td>JY</td>
<td>4.45 ± 0.08</td>
<td>0.42</td>
<td>169.0 ± 12.0</td>
<td>47.0</td>
<td>112</td>
</tr>
<tr>
<td>CEM</td>
<td>2.68 ± 0.28</td>
<td>0.27</td>
<td>12.8 ± 0.04</td>
<td>3.6</td>
<td>13</td>
</tr>
</tbody>
</table>

* Direct saturation binding was determined as described in Materials and Methods by titration of ^51^Cr-labeled TS1/18.1.1 (anti-LFA-1, specific activity 2.1 x 10^6 cpm/μg) or ^51^Cr-labeled MST target cells (anti-HLA, specific activity 2.8 x 10^6 cpm/μg) on 2 x 10^3 or 5 x 10^3 cells, respectively.

**TABLE II**

Comparison of CTL-inhibiting activity and antigenic epitopes of anti-human LFA-1 monoclonal antibodies

<table>
<thead>
<tr>
<th>Anti-LFA-1 Monoclonal Antibody</th>
<th>% Inhibition of Cytolysis</th>
<th>Epitope Reactivity</th>
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<tbody>
<tr>
<td>TS1/18</td>
<td>51 ± 14</td>
<td>unique</td>
</tr>
<tr>
<td>TS2/22</td>
<td>40 ± 11</td>
<td>shared (14,4,18,6,11)</td>
</tr>
<tr>
<td>TS2/6</td>
<td>35 ± 10</td>
<td>shared (18,11)</td>
</tr>
<tr>
<td>TS2/14</td>
<td>29 ± 3</td>
<td>unique</td>
</tr>
<tr>
<td>TS1/11</td>
<td>12 ± 5</td>
<td>unique</td>
</tr>
<tr>
<td>TS2/4</td>
<td>6 ± 6</td>
<td>shared (6,11,18,22)</td>
</tr>
</tbody>
</table>

* The percentage of inhibition of cytolysis was calculated as a ratio of the % lysis in the presence of 3 μg/ml of the indicated MAb to the % lysis in medium alone during a 4-hr ^51^Cr-release assay. The data represent the mean ± SD of several different experiments using CTL-11 line on JY or MST target cells at 10:1 to 20:1 effector:target ratio. n = 14 for TS1/18; n = 2 to 4 experiments for the other LFA-1 MAb.

**DISCUSSION**

The studies presented here have investigated the immunological properties of the human LFA-1 molecule, quantitated LFA-1 expression, and described the topographic relationship of the antigenic epitopes and the functional site(s) of human LFA-1 in comparison with the epitope topography of mouse LFA-1. Three unique and three complex epitopes on the human LFA-1 molecule (summarized in Table II) were identified with Balb/c-derived IgG, monoclonal antibodies by using a competitive cross inhibition binding assay (Fig. 1). Immunoprecipitation precloning studies indicated the epitopes resided on the same LFA-1 molecule (Fig. 2). Several rat anti-mouse LFA-1 MAb similarly tested showed significant competitive cross-inhibition, and thus by this criteria, identify a common determinant and one partially shared epitope on mouse LFA-1 (Fig. 3). These rat anti-mouse LFA-1 MAb were derived from several fusions representing an extensive search for CTL inhibiting MAb (6). In addition, several other rat anti-LFA-1 MAb derived in other laboratories (25, 26) were also competitive inhibitors of the M7/14 MAb [T. A. Springer unpublished observations, (47)]. These results indicate that cytolytically inhibiting rat MAb recognize a very limited number of epitopes on mouse LFA-1. The antigenic diversity observed with human LFA-1 as defined by mouse MAb most likely reflects the species diversity between the immunized host and the eliciting antigen. This suggests that xenogenic immunization with human cells will be useful in obtaining MAb which recognize highly conserved molecules involved in CTL function. This suggestion finds additional support by the discoveries of the T3, LFA-2 and LFA-3 antigens in man; their equivalents, as of yet, have not been found in the mouse even though extensive studies have been conducted (5, 6).

Quantitation of LFA-1 sites on various cell types revealed that a cytolytically active human CTL line contained 2 to 4 times more...
LFA-1 than normal PBL or T and B lymphoblastoid cell lines (Table I). HLA-A, B, C antigen expression was 20-fold higher on a B lymphoblastoid cell than on PBL, while these cells expressed similar levels of LFA-1. In the mouse, T cells were found to express higher levels than B cells, and activated mouse T lymphocytes (primary MLC-sensitized CTL and Concanavalin A blasts) were found to express significantly higher levels of LFA-1 than normal splenocytes (48). This may be analogous to the higher level of LFA-1 expression observed on human CTL-11 line compared to PBL. Collectively, these data indicate that LFA-1 expression is quantitatively increased on functionally active lymphocyte populations and suggest that higher LFA-1 expression may be related to a gain in T cell effector function.

The studies presented here provide several important results germane to the mechanism of anti-LFA-1 MAb inhibition of human CTL-mediated cytotoxicity. The identification of several unique epitopes on human LFA-1 provides, in part, an explanation for the variability in the inhibitory activity of the TS1 and TS2 anti-human LFA-1 MAb. All of these MAb are of the \(\gamma_1\), chain subclass and thus antibody subclass differences would not account for the differential blocking activities. Differences in individual MAb affinity constants may have contributed to this variability although this seems unlikely since the concentrations of MAb used in the inhibition studies were in considerable excess of saturation (100 ng TS1/18 is 50% saturating for 2.5 \times 10^6 cells; 1-3 \mu g/ml was required to maximally inhibit 10^6 CTL cells) and the anti-LFA-1 MAb are of sufficiently high avidity to immunoprecipitate LFA-1.

The ability of anti-LFA-1 MAb to inhibit CTL function cannot be attributed to trivial means but appears to be through a specific interaction with the effector cell surface LFA-1 molecules. Anti-LFA-1 MAb did not agglutinate nor was it directly cytotoxic to CTL as evidenced after staining of treated cells with the vital dye trypan blue (C. Ware, unpublished) and the effects of anti-LFA-1 were largely reversible within a short time (Fig. 6). Although the B lymphoblastoid target cells express LFA-1, studies to be presented elsewhere (Krensky et al., manuscript in preparation) have shown that pretreatment of the CTL population with anti-LFA-1 MAb will block CTL function, i.e., precoccygation of the target cell was not sufficient to block lysis. The relative inability of the TS2/4 MAb to inhibit CTL function provides an excellent specificity control for blocking effects of the anti-LFA-1 MAb. Of interest to note, the TS2/4 MAb should prove to be an excellent reagent for cytochemical localization of LFA-1 during CTL-target cell interactions without perturbation of LFA-1 biological activity.

The high density of HLA relative to LFA-1 (10:1) on the CTL surface coupled with the fact that anti-HLA antibodies such as W6/32 when bound to the effector CTL do not block the cytolitic reaction, even though they do when bound to the target cell seems unlikely since the concentrations of MAb used in the inhibition studies were in considerable excess of saturation (100 ng TS1/18 is 50% saturating for 2.5 \times 10^6 cells; 1-3 \mu g/ml was required to maximally inhibit 10^6 CTL cells) and the anti-LFA-1 MAb are of sufficiently high avidity to immunoprecipitate LFA-1.

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**REFERENCES**

IMMUNOCHEMISTRY OF HUMAN LFA-1

Protein antigen and receptor interactions are critical for many biological processes, including cell-cell interactions, receptor-mediated endocytosis, and immune responses. This chapter focuses on the immunoprecipitation of T cell surface polypeptides by monoclonal antibodies, which has provided insights into the structure and function of the CD11a/CD18 complex, also known as LFA-1 (Lymphocyte Function-Associated Antigen 1).

Monoclonal antibodies against LFA-1 have been instrumental in studying the interactions between human T lymphocytes and various cell types, including endothelial cells and other immune cells. These interactions play a crucial role in cell-mediated immunity and inflammation.


